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(54) Title: PRODUCT AND PROCESS FOR T CELL REGULATION

(57) Abstract

The present invention relates to a product and process for regulating the activity of T cells using major histocompatibility complexes (MHC) stably linked to antigenic peptides. Disclosed is an antigenic peptide covalently linked to a major histocompatibility complex (MHC) protein by a novel linker, thereby enabling the formation of a stable peptide-MHC complex, alone or in combination with additional MHC protein chains, capable of being recognized by a T cell receptor (TCR). Also disclosed is a nucleic molecule having a sequence encoding a Peptide-L-MHC molecule comprising an antigenic peptide joined by a linker to an MHC segment. The invention is additionally directed to formulations comprising an antigenic peptide joined by a linker to an MHC segment anchored in a lipid-containing substrate. Pharmaceutical reagents are also disclosed which contain an antigenic peptide joined by a linker to an MHC segment combined with a suitable carrier that is capable of presenting the Peptide-L-MHC molecule so that it is capable of being recognized by a T cell receptor.

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PRODUCT AND PROCESS FOR T CELL REGULATION

Field of the Invention

The present invention relates to a product and process for regulating the activity of T cells using major histocompatibility complexes (MHC) stably linked to antigenic peptides.

Background

A wide variety of medical treatments require regulation of the immune response in a patient. Such treatments include, for example, treatments for autoimmune diseases, immunodeficiency diseases, immunoproliferative diseases, and treatments involving the transplantation of organs and skin. Traditional reagents and methods used to regulate an immune response in a patient often result in unwanted side effects. For example, immunosuppressive reagents such as cyclosporin A, azathioprine, and prednisone are used to suppress the immune system of a patient with an autoimmune disease or patients receiving transplants. Such reagents, however, suppress a patient's entire immune response, thereby crippling the ability of the patient to mount an immune response against infectious agents not involved in the original disease. Due to such harmful side effects and the medical importance of immune regulation, reagents and methods to regulate specific parts of the immune system have been the subject of study for many years.

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mixing in solution the isolated MHC protein with purified peptides. Separation of the MHC protein is a laborious, multi-step process and requires a large number of cells to obtain sufficient amounts of MHC protein. In addition, considerable effort is required to obtain purified peptide to mix with the MHC protein. Moreover, the association between individual peptides and MHC has been shown to be unstable.

Particular MHC-peptide complexes and methods of making them have been suggested by various investigators, including Clark et al., U.S. Patent No. 5,260,422, issued November 9, 1993; Sharma et al., U.S. Patent No. 5,194,425, issued March 16, 1993; Sharma et al., U.S. Patent No. 5,130,297, issued July 14, 1992; Nag et al., PCT Application No. WO 93/09810, published May 27, 1993; and Sanderson, U.S. Application No. 4,400,376, published Aug. 23, 1983. Prior investigators, however, have only disclosed the use of soluble, as opposed to membrane bound, MHC-peptide complexes. Moreover, methods to produce such complexes suffered from the unpredictable and unstable association of peptides with MHC.

As such, there is a need for a product and process that allows for the cost-effective production of large quantities of both soluble and membrane bound MHC-peptide complexes wherein the peptides are stably associated with the MHC proteins.

Summary

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The present invention is also directed to formulations comprising Peptide-L-MHC molecules combined with suitable carriers such that the formulations are capable of effecting an immune response. Such formulations are useful as pharmaceutical reagents for the treatment of diseases including autoimmune diseases, immunostimulatory diseases, immunoproliferation diseases and graft-host rejection. The formulations are also useful as experimental reagents. In one embodiment, compositions and molecules of the present invention are anchored to the plasma membranes of cells that are incapable of stimulating a T cell response. Cells incapable of stimulating a T cell response include red blood cells, fibroblasts, pluripotent progenitor cells, epithelial cells and neural cells.

In another embodiment, compositions and molecules of the present invention are anchored in plasma membranes of cells that are capable of stimulating a T cell response. Cells capable of stimulating a T cell response include macrophages, B cells and dendritic cells.

One embodiment of the present invention includes Peptide-L-$MHC_{\alpha+\beta 2m}$ compositions anchored to the plasma membrane of cells that are capable of stimulating a T cell response and to cells that are incapable of stimulating an immune response. Another embodiment of the present invention includes Peptide-L-$MHC_{\alpha+\beta}$ compositions anchored to the plasma membrane of cells that are capable of

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Fig. 1 is a schematic representation of a peptide covalently associated with the binding site of an MHC protein a linker.

Fig. 2 illustrates the stabilization of soluble MHC class II molecules by covalently attached peptides.

Fig. 3 illustrates the stimulation of T cell hybridomas by purified, immobilized class II protein bound by an antigenic peptide by a linker.

Fig. 4 illustrates the expression of IA^b-Ea protein by M12.C3 cells.

Fig. 5 illustrates the inhibition by covalently associated peptide of OVA and ovalbumin to IA^b-Ea protein expressed on M12.C3 cells.

Fig. 6 illustrates stimulation of T cell hybridomas IA^b-Ea protein bearing M12.C3 cells.

Fig. 7 illustrates the expression of IA^b-Ea protein on fibroblast cells.

Fig. 8 illustrates the inhibition by covalently associated peptide of OVA binding to IA^b-Ea protein expressed on fibroblast cells.

Fig. 9 illustrates stimulation of T cell hybridomas by IA^b-Ea bearing fibroblast cells.

Fig. 10 illustrates that the IE^k-MCC transgene is expressed in bone marrow and spleen populations.

Fig. 11 illustrates that the IE^k-MCC transgene is expressed in Ter 119 antigen positive red blood cells.

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Peptide-L-MHC$_{\alpha+\beta}$ composition as described in detail below. A representation of an antigenic peptide bound to a binding site of an MHC protein and covalently associated with the MHC protein is shown in Fig. 1.

The major histocompatibility complex is a collection of genes encoding glycoproteins called major histocompatibility complex (MHC) proteins. *In vivo*, the primary function of an MHC protein is to present antigen in a form capable of being recognized by a TCR. An MHC protein is bound to an antigen in the form of an antigenic peptide to form an MHC-peptide complex. As used herein, "MHC-peptide complex" refers to any MHC protein having an antigenic peptide bound to one or more of the MHC protein's peptide binding sites.

As used herein, "TCR recognition" refers to the ability of a TCR to bind to an MHC peptide complex. The presentation of antigen by an MHC protein to the T cell normally leads to a T cell response that is clone specific. Normal T cells are distinguished from T cell hybridomas which may differ from normal T cells in their activation reactions. As used herein, "antigen presentation" refers to presenting antigen in such a manner that at least a portion of the antigen is available to be bound by a TCR. A T cell response occurs when a TCR recognizes an MHC protein bound to an antigenic peptide, thereby altering the activity of the T cell bearing the TCR. As used herein, a "T cell response" can refer to the activation, induction of anergy, or death of a T cell that

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intracellular domains (a TM domain and a CYT domain). The β chain contains two extracellular domains (β_1 and β_2), and a TM and CYT domain.

Antigenic peptides associate with an MHC protein by binding to a peptide binding site of an MHC protein. As used herein, the term "peptide binding site" refers to the portion of an MHC protein capable of binding peptide. Peptide binding sites can be internal binding sites (e.g., peptide binding grooves) or external binding sites (e.g., binding sites on the external surface of an MHC protein). The conformation of a peptide binding site is capable of being altered upon binding of an antigenic peptide to enable proper alignment of amino acid residues important for TCR binding to the MHC protein and/or peptide.

The domain organization of class I and class II proteins form the peptide binding site. In one embodiment of the present invention, a peptide binding site includes a peptide binding groove. A peptide binding groove refers to a portion of an MHC protein which forms a cavity in which a peptide can bind. A peptide binding groove of a class I protein can comprise portions of the α_1 and α_2 domains. A binding groove of a class II protein can comprise portions of the α_1 and β_1 domains capable of forming two β-pleated sheets and two α helices. Without being bound by theory, it is believed that a first portion of the α_1 domain forms a first β-pleated sheet and a second portion of the α_1 domain forms a first α helix. A first portion of the β_1 domain forms a second β-pleated

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class II protein includes: the α_1 domain or α_2 domain of the α chain; the β_1 domain or β_2 domain of the β chain; or a combination of these domains.

In yet another embodiment, a binding site can also comprise a "combined binding site" having portions of an external binding site and portions of a binding groove which can be bound by an antigenic peptide.

One embodiment of the present invention is a Peptide-L-MHC molecule, the description of which can be best conveyed by individually discussing the various components of the novel Peptide-L-MHC. An MHC segment of the present invention can be any portion of an MHC protein that is sufficient to form, either alone or in combination with the appropriate portion of an MHC protein chain, a peptide binding site capable of presenting antigenic peptide in a manner that is able to be recognized by a TCR.

In one embodiment, an MHC segment of a Peptide-L-MHC molecule of the present invention can comprise at least a portion of a class I MHC protein, at least a portion of a class II MHC protein, or a hybrid thereof. As used herein, a "hybrid" refers to the attachment of at least a portion of a class I MHC protein to at least a portion of a class II MHC protein, to form a single MHC functional protein. According to the present invention, "at least a portion" refers to a portion of an MHC protein capable of forming a peptide binding site or capable of forming a binding site upon addition of another chain of an MHC protein. Preferred MHC segments of the present invention

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least one MHC transmembrane domain and at least a portion of at least one MHC cytoplasmic domain.

In another embodiment, an MHC segment of the present invention can include at least a portion of a single chain such as a class I α chain; a class II α chain; a class II β chain; or hybrids thereof. Hybrids can include any combination of such portions, such as a single chain comprising a portion of a class I α chain attached to a portion of a class II β chain. Peptide-L-MHC molecules containing such MHC segments can be combined with an appropriate distinct MHC protein chain capable of associating with the Peptide-L-MHC molecule to form a complex having a function peptide binding site.

A preferred class I α chain of the present invention contains class I α_1, α_2 and α_3 domains. A preferred class II α chain of the present invention contains class II α_1 and α_2 domains. A preferred class II β chain contains β_1 and β_2 domains. A preferred hybrid MHC segment of the present invention includes at least portions of a class I α chain and a class II β chain.

Preferred embodiments of an MHC segment of the present invention include segment having a class II β chain which includes a β_1 domain, a β_2 domain, and a segment having a class II β chain which includes a β_1 domain, a β_2 domain, a β chain transmembrane domain and a β chain cytoplasmic domain.

An antigenic peptide of the present invention can comprise any peptide that is capable of binding to an MHC

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proteins in extracellular fluids such as serum. The MHC-peptide complex can then be presented to a TCR. In another embodiment, antigenic peptides that are not hydrolyzed can associate with unoccupied MHC proteins on the surface of a T cell and can be presented to a TCR.

Another embodiment of the present invention is a groove-binding antigenic peptide, which is an antigenic peptide that is capable of binding to a peptide binding groove of an MHC protein in such a manner that the resulting Peptide-MHC complex can bind to a TCR. It is believed that the binding of an antigenic peptide to an MHC peptide binding groove can control the spatial arrangement of MHC and/or antigenic peptide amino acid residues recognized by a TCR. Such spatial control may be due in part to hydrogen bonds formed between a peptide and an MHC protein. Preferably, the length of a groove-binding antigenic peptide extends from about 5 to about 40 amino acid residues, more preferably from about 6 to about 30 amino acid residues, and even more preferably from about 8 to about 20 amino acid residues.

Preferred groove-binding antigenic peptides include those that bind to MHC protein involved in autoimmune diseases, immunodeficiency diseases, immunoproliferation diseases, and graft-host rejection. More preferred groove specific peptides of the present invention include Arg-Ala-Asp-Leu-Ile-Ala-Tyr-Leu-Lys-Gln-Ala-Thr-Lys, Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg, Arg-Ala-Asp-Leu-Ile-Ala-Tyr-Leu-Lys-Gln-Ala-Thr-Lys, Val-His-Ala-Ala-

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multiple sclerosis, and insulin dependent diabetes mellitus.

Preferred infectious agents of the present invention include, but are not limited to, bacteria, viruses, and eukaryotic parasites. Preferred animal parasites include protozoan parasites, helminth parasites (such as nematodes, cestodes, trematodes, ectoparasites and fungi.

Preferred allergens of the present invention include, but are not limited to plant, animal, bacterial, parasitic allergens and metal-based allergens that cause contact sensitivity.More preferred allergens include weed, grass, tree, peanut, mite, flea, and cat antigens.

Preferred toxins of the present invention include, but are not limited to, staphylococcal enterotoxins, toxic shock syndrome toxin, retroviral antigens, streptococcal antigens, mycoplasma, mycobacterium, and herpes viruses. Retroviral antigens include antigens derived from human immunodeficiency virus. Even more preferred toxins include SEA, SEB, SE_{1-3}, SED, and SEE.

A Peptide-L-MHC molecule of the present invention contains a novel linker which comprises an amino acid sequence that covalently associates an MHC segment with an antigenic peptide. Covalent bonds are formed between the antigenic peptide and the linker, and between the linker and the MHC segment. The linker is distinguished from a peptide linkage which refers to the chemical interaction between two amino acids.

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interactions between a linker and an MHC protein. A skilled artisan would also predict that a linker would hinder the interaction of an MHC protein bound by antigenic peptide with a TCR by stearic hindrance and/or amino acid charge interactions. Despite such prevalent beliefs in the field, the present inventors have identified and produced linkers that do not substantially hinder the association of an antigenic peptide with an MHC binding site and, moreover, stabilize the association of the peptide with the MHC.

A linker useful in the production of a Peptide-L-MHC molecule can comprise any amino acid sequence that facilitates the binding of an antigenic peptide to an MHC protein. A linker can facilitate antigenic peptide binding by, for example, maintaining an antigenic peptide within a certain distance of an MHC peptide binding site to promote efficient binding. The linker enhances the ability of a combined aggregate of antigenic peptide and MHC protein to act as a unit in triggering a desired immune response. Preferably, a linker of the present invention is capable of facilitating the binding of the antigenic peptide portion of a Peptide-L-MHC molecule to the MHC protein segment of either a Peptide-L-$MHC_{\alpha+\beta}$ composition or a Peptide-L-$MHC_{\alpha+\beta 2m}$ composition (as defined in detail below) that is capable of being recognized by a TCR.

According to the present invention, a linker of the present invention stabilizes the association of an

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A linker useful in the production of a Peptide-L-MHC molecule can comprise any amino acid sequence that does not substantially hinder interaction of an antigenic peptide with an MHC protein or hinder interaction of an MHC protein bound by peptide with a TCR.

The length of a linker of the present invention is preferably sufficiently short (i.e., small enough in size) such that the linker does not substantially inhibit binding between the antigenic peptide and the MHC segment of a Peptide-L-MHC molecule or inhibit TCR recognition. Preferably, the length of a linker of the present invention is from about 1 amino acid residue to about 40 amino acid residues, more preferably from about 5 amino acid residues to about 30 amino acid residues, and even more preferably from about 8 amino acid residues to about 20 amino acid residues.

In addition, the amino acid composition of a linker of the present invention is substantially neutral such that the linker does not inhibit MHC-peptide complex formation or TCR recognition by the complex. As used herein, the term "neutral" refers to amino acid residues sufficiently uncharged or small in size so that they do not prevent interaction of a linker with an MHC segment. Preferred amino acid residues for linkers of the present invention include, but are not limited to glycine, alanine, leucine, serine, valine, threonine, and proline residues. More preferred linker amino acid residues include glycine, serine, leucine, valine, and proline

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TCR. A processable linker can further comprise an immunogenic sequence representing a foreign determinant that can be removed by cleaving serum enzyme target sites also contained on the linker.

Preferred processable linkers of the present invention include linkers containing target sites for enzymes such as collagenases, metalloproteases, serine proteases, cysteine proteases, kallikriens, thrombin, and plasminogen activators. A preferred processable linker of the present invention includes a linker having a thrombin cleavage site of Leu-Val-Pro-Arg-Gly-Ser.

Suitable linkers useful in the present invention can be designed using various methods. For example, x-ray crystallographic data of an MHC protein can be used to design a linker of suitable length and charge such that the linker does not interfere with TCR recognition of the MHC-peptide complex. Suitable linkers can also be identified by producing large numbers of different Peptide-L-MHC molecules having different combinations of antigenic peptides, linkers and MHC segments and determining if those molecules, alone or in combination with other distinct MHC protein chains, can be recognized by a TCR. In addition, linkers know to function well with one particular Peptide-L-MHC molecule can be combined with other antigenic peptide and MHC segment combinations, and tested for the ability of the resulting Peptide-L-MHC molecule to affect a T cell response, either alone or in combination with other MHC protein chains.

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toxin, *Pseudomonas* exotoxin, modeccin toxin, abrin toxin, and shiga toxin; single-chain toxins, such as pokeweed antiviral protein, alpha-amanitin, and ribosome inhibiting proteins; and chemical toxins, such as melphalan, methotrexate, nitrogen mustard, doxorubicin and daunomycin. Suitable labels include, but are not limited to fluorescent labels, biotin, at least a portion of an immunoglobulin protein, metallic compounds, luciferin, radiolabels and enzymes.

Another aspect of the present invention relates to a nucleic acid molecule that encodes a protein of the present invention comprising the Peptide-L-MHC molecules disclosed herein. According to the present invention, references to nucleic acids also refer to nucleic acid molecules. A nucleic acid molecule can be DNA, RNA, or hybrids or derivatives of either DNA or RNA. Nucleic acid molecules of the present invention can include regulatory regions that control expression of the nucleic acid molecule (e.g., transcription or translation control regions), full-length or partial coding regions, and combinations thereof. Any portion of a nucleic acid molecule of the present invention can be produced by: (1) isolating the molecule from its natural milieu; (2) using recombinant DNA technology (e.g., PCR amplification, cloning); or (3) using chemical synthesis methods. A nucleic acid of the present invention can include functional equivalents of natural nucleic acid molecules encoding an MHC segment or a peptide, including, but not

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modifications to nucleic acid sequences would be appropriate and which would not.

One embodiment of the present invention includes a nucleic acid molecule encoding a Peptide-L-MHC molecule having at least three components: (1) an MHC segment; (2) a peptide; and (3) a linker. Suitable and preferred segments, peptides and linkers for use in the present invention are heretofore disclosed. A nucleic acid molecule of the present invention comprises at least one nucleic acid sequence encoding an MHC segment, covalently attached (by base pair linkage) to at least one nucleic acid sequence encoding a linker, which is itself covalently attached (by base pair linkage) to at least one nucleic acid sequence encoding an antigenic peptide. The nucleic acid sequences are attached in such a manner that the sequences are transcribed in-frame, thereby producing a functional Peptide-L-MHC molecule capable of forming a peptide binding site, alone or in combination with another MHC protein chain.

Preferred nucleic acid molecules encoding Peptide-L-MHC molecules include: nucleic acid sequences encoding an MHC class I α chain, a linker and an antigenic peptide, wherein the peptide is linked to the class I α chain by the linker; and an MHC class II β chain, a linker and an antigenic peptide, wherein the peptide is linked to the class II β chain by the linker. Particularly preferred nucleic acid molecules encode IEβ^k or a functional equivalent thereof; IAβ^d or a functional equivalent

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a separate nucleic acid molecule as the sequence encoding an MHC class II β chain. For example, a nucleic acid molecule encoding a Peptide-L-$MHC_{\alpha+\beta}$ composition can contain: a sequence encoding a MHC class I MHC_{α}-L-Peptide molecule ligated to a sequence encoding a β2m subunit; a sequence encoding an MHC class II MHC_{β}-L-Peptide molecule ligated to a sequence encoding an MHC class II α chain encoding sequence; or a sequence encoding an MHC class II MHC_{α}-L-Peptide molecule ligated to a sequence encoding an MHC class II β chain encoding sequence.

In other embodiments, a nucleic acid sequence is used that encodes for a signal or leader segment that is capable of promoting secretion of a Peptide-L-MHC molecule from the cell that produces the molecule. Nucleic acid sequences encoding the leader or signal segments are covalently associated (by base pair linkage) to the 5' end of a nucleic acid molecule. The leader or signal segments can be segments which naturally are associated with an MHC segment or are heterologous. Preferred segments are naturally associated segments. To obtain membrane-bound embodiments, nucleic acid sequences are used that contain at least one transmembrane segment capable of anchoring a Peptide-L-MHC molecule to a lipid-containing substrate, such segments including at least a portion of an MHC transmembrane domain and at least a portion of an MHC cytoplasmic domain. A nucleic acid sequence encoding a transmembrane segment is covalently associated (by base pair linkage) to the 3' end of a nucleic acid sequence

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molecules. Fusion proteins are preferably produced by culturing a recombinant cell transformed with a fusion nucleic acid sequence that encodes a protein including the fusion segment attached to either the carboxyl and/or amino terminal end of a Peptide-L-MHC molecule.

Particularly preferred nucleic acid molecules include: N-IE^{kd}-MCC having sequences encoding a moth cytochrome C (91-103) peptide, a linker and an IEβ^k protein (described in detail in Example 1); N-IA^d-OVA having sequences encoding the cOVA (327-339) peptide, a linker and an IAβ^d protein (described in detail in Example 1); and N-IA^b-Ea having sequences encoding an IEα^d (56-73) peptide, a linker and an IAβ^b protein (described in detail in Example 2).

The present invention also includes a recombinant molecule comprising a nucleic acid sequence encoding a Peptide-L-MHC molecule operatively linked to a vector capable of being expressed in a host cell. As used herein, "operatively linked" refers to insertion of a nucleic acid sequence into an expression vector in such a manner that the sequence is capable of being expressed when transformed into a host cell. As used herein, an "expression vector" is an RNA or DNA vector capable of transforming a host cell and effecting expression of an appropriate nucleic acid sequence, preferably replicating within the host cell. An expression vector can be either prokaryotic or eukaryotic, and typically is a virus or a plasmid.

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baculovirus transfer vectors, and vectors containing class II promoters, β-actin promoters, globin promoters, or epithelial cell specific promoters.

An expression system can be constructed from any of the foregoing control elements operatively linked to the nucleic acid sequences of the present invention using methods known to those of skill in the art. (see, for example, Sambrook et al., *ibid.*)

Host cells of the present invention can be: cells naturally capable of producing MHC protein; or cells that are capable of producing MHC protein when transfected with a nucleic acid molecule encoding an MHC protein. Host cells of the present invention include, but are not limited to bacterial, fungal, insect and mammalian cells. Suitable host cells include mammalian cells capable of stimulating a T cell response, preferably antigen presenting cells including dendritic cells, macrophages and B lymphocytes, as well as cells that are not capable of stimulating a T cell response, preferably fibroblasts, red blood cells, pluripotent progenitor cells, epithelial cells and neural cells.

One particular embodiment involves a host cell transformed with a recombinant molecule encoding a Peptide-L-MHC molecule, wherein the MHC segment of the molecule is a class II β chain. The host cell can also be co-transformed with a recombinant molecule encoding an MHC class II α chain capable of associating with the Peptide-L-MHC molecule to form a Peptide-L-$MHC_{\alpha+\beta}$ composition.

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Soluble Peptide-L-MHC molecules of the present invention can be purified using, for example, immunoaffinity chromatography. Peptide-L-MHC molecules anchored in a lipid-containing substrate can be recovered by, for example, density gradient centrifugation techniques.

One aspect of the present invention relates to the use of Peptide-L-MHC molecules and nucleic acid molecules of the present invention as formulations for therapeutic or experimental use. In one embodiment, a Peptide-L-MHC molecule of the present invention can be used to produce a protein pharmaceutical reagent. Such protein pharmaceutical reagents are useful for administration to patients suffering from diseases such as autoimmune diseases, immunodeficiency diseases, and immunoproliferative diseases, or from graft-host rejection. A protein pharmaceutical reagent includes a Peptide-L-MHC molecule associated with a suitable carrier. A Peptide-L-MHC molecule of the present invention can also be used to produce a protein experimental reagent. A protein experimental reagent is a reagent useful for the development of drugs and for the study of different aspects of an immune response. A protein experimental reagent includes a Peptide-L-MHC molecule associated with a suitable pharmaceutically acceptable carrier. "Pharmaceutically acceptable" refers to a compound that is not harmful (e.g., toxic) to a cell or an animal.

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lauric, palmitic, stearic, linoleic, linolenic, olesteric, and oleic acids. Formulations of the present invention can be sterilized by conventional methods and/or lyophilized.

Carriers of the present invention can also include adjuvants including, but not limited to, Freund's adjuvant; other bacterial cell wall components; aluminum-based salts; calcium-based salts; silica; polynucleotides; toxoids; serum proteins; viral coat proteins; and other bacterial-derived preparations.

Useful carriers for membrane-bound Peptide-L-MHC molecules include any artificial or natural lipid-containing substrate, preferably cells, cellular membranes, liposomes and micelles. Cellular carriers of the present invention include cells essentially incapable of stimulating T cells , such as cells that lack secondary proteins capable of mediating T cell activation, as well as cells that stimulate T cells and that have secondary proteins that mediate T cell activity. Preferred mammalian cells of the present invention include, but are not limited to antigen presenting cells, fibroblasts, red blood cells, pluripotent progenitor cells, epithelial cells, and neural cells. Antigen presenting cells are cells that typically express MHC proteins on their cell surface and that are capable of processing antigens. Preferred antigen presenting cells include, for example, dendritic cells, macrophages and B lymphocytes.

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binding to B7 in the presence of TCR binding by a peptide-MHC complex, does not result in activation of a T cell.

Preferred lipid-containing carriers for protein formulations capable of suppressing T cell activity include, for example micelles, liposomes, cells and cellular membranes essentially incapable of stimulating a T cell response. More preferred carriers include mammalian cells, such as red blood cells, fibroblast cells, pluripotent progenitor cells, epithelial cells and neural cells.

In another embodiment, a membrane-bound Peptide-L-MHC molecule is used to produce protein formulations capable of stimulating T cell activity. As used herein, T cell stimulation refers to the activation of a T cell resulting in biological function, such as IL-2 production or cytotoxic activity. Preferably, lipid-containing carriers, such as micelles, liposomes, cells and cellular membranes essentially capable of stimulating a T cell response, are used to produce formulations that are capable of stimulating T cell activity. More preferred carriers include mammalian antigen presenting cells, such as dendritic cells, macrophages and B lymphocytes.

In yet another embodiment, a soluble Peptide-L-MHC molecule is used to produce protein formulations capable of suppressing T cell activity. Preferred carriers for soluble Peptide-L-MHC molecules include physiologically balanced solutions, and a more preferred carrier is phosphate buffered saline.

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The reocmbinant molecule used in gene therapy can be those that do not integrate into the genome of the a host cell or those that do integrate. Recombinant molecules that do integrate into the genome of a cell are particularly useful for introduction into pluripotent cells, such as stem cells or pluripotent hematopoietic cells. Recombinant molecules that do not integrate into the genome of a host cell are particularly useful for introduction into lineage-committed cells (e.g., immature or mature lymphocytes, erythrocytes and leukocytes). Recombinant molecules that do not integrate into the genome of a host cell can be non-replicating DNA sequences, or specific replicating sequences genetically engineered to lack the genome-integration ability.

Specific promoters can be employed in gene therapy applications based upon the type of cell being transformed. Thus, inducible production of a desired product encoded by transformed genes can be achieved. Suitable promoters for use in various vertebrate systems include those promoters disclosed in detail herein, In humans, a particularly useful promoter includes a cytomegolavirus IEP promoter.

Recombinant molecules can be delivered to an animal in a variety of methods including, but not limited to, (a)direct injection (e.g., as "naked" DNA or RNA molecules, such as is taught, for example in Wolff et al., 1990, *Science 247*, 1465-1468) or (b) packaged as a recombinant virus particle vaccine or as a recombinant

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Such diseases include autoimmune diseases, immunodeficiency diseases, and immunoproliferative diseases. A pharmaceutical reagent of the present invention is also useful for treatments involving the transplantation of organs and skin. Autoimmune diseases include, for example, systemic lupus, myasthenia gravis, rheumatoid arthritis, insulin dependent diabetes mellitus and experimental allergic encephalomyelitis. Immunodeficient diseases include, for example, human AIDs, hypogammaglobulinemia, DiGeorge Syndrome, chronic mucocutaneous candidiasis, GVH disease, combined immunodeficiency disease, Nezelof's Syndrome, episodic lymphopenia, and immunodeficiencies related to thymomas, eczema, thrombocytopenia, adenosine diaminase deficiency and dwarfism. Immunoproliferative diseases include, for example, lymphomas and leukemias. In addition, a pharmaceutical reagent of the present invention capable of stimulating a T cell response is useful for the treatment of specific disorders such as tumors, allergic responses and inflammation.

Experimental reagents of the present invention includes a protein formulation useful for, for example, screening for peptides capable of regulating T cell activity, antibodies that bind MHC protein complexed with peptide, for TCR's capable of binding MHC protein complexed with peptide, and for T cells bearing TCR capable of binding MHC protein complexed to peptide.

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chain (IAß^d) protein, were prepared using polymerase chain reaction (PCR) amplification of cloned cDNA templates. To allow for secretion of fully assembled $\alpha\beta$ dimers, these genes were truncated at the last codon prior to codons encoding the transmembrane and cytoplasmic portions of the proteins. Primers used for PCR amplification of the IEα^d gene were 5' TCCTCGAGAAATGGCCACAATTGGAG 3' and 3' CTTTGATTTCTCTTAATTCCATGGTT 5'. Primers used for PCR amplification of the IEß^k gene were 5' CCGGGAATTCAGCATGGTGTGGCTCC 3' and 3' AGACGTTCTTGTTGCATTCGTACGCC 5'. The alignment of the primers with their respective cDNA templates is shown in Table 1.

Genes encoding IAα^d and IAß^d proteins were also produced by PCR amplification. Primers used for PCR amplification of the IAα^d gene were 5' TCCTCGAGAGGATGCCGTGCAGCAGAG 3' and 3' CTCGACTGGTCTTTGAATTCCATGGTT 5'. Primers used for PCR amplification of the IAß^d gene were 5' TACGGAATTCTTAGAGATGGCTCTGCAGA 3' and 3' TCAGACGGGCCTCGTTCATTCGTACGCC 5'. The alignment of the primers with their respective cDNA templates is shown in Table 1. The restriction enzyme sites used for cloning the IEα^d, IEß^k, IAα^d and IAß^d genes into new multiple cloning sites (MCS) of a baculovirus transfer vector are also shown in Table 1.

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Using PCR amplification, a hybrid IEß^k nucleic acid molecule was produced containing sequences encoding amino acid residues 91-103 of moth cytochrome c (MCC Peptide (91-103), a linker containing a thrombin cleavage site, and the IEß^k gene. The hybrid molecule was prepared as follows. Referring to Table 2, a first fragment (fragment 234-328) was produced encoding the leader and the first three codons of the β1 domain of IEß^k, and the first 11 codons of the MCC peptide using Primer #234 (5' TACGGAATTCAGCATGGTGTGGCTCCC 3') and Primer #328 (5'ACCGGACGAAGTTTATCCGTTAGTCCAGTCGGGCCCTCAGAGACTGGTT 3') on an IEß^k cDNA template. Primer #234 contains an EcoRI

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restriction enzyme site and Primer #328 contains an XmaI restriction enzyme site. Fragment 234-328 was used as a template for a second PCR fragment (fragment 234-329) in which fragment 234-328 was extended by adding sequences encoding the remainder of the MCC peptide and the first 10 codons of the linker were added using Primer #329 (5' GGGGCACCGTGATCACTCGGTGGTGGAGGGAACCACCGGACGAAGTTTAT 3'). Primer #329 contains an SpeI site. Fragment 234-329 was used as a template for a third PCR fragment (fragment 234-330) in which fragment 234-329 was extended by adding sequences encoding the remainder of the linker and residues 4-8 of the β1 domain using Primer #330 (5' TTTTTGGTACCAGACCTGGGTGGAGGAGGTCTCGGGGCACCGTGATCACTCG 3'). Primer #330 contains a SpeI site and an NcoI site. Fragment 234-330 was then digested with EcoRI and NcoI and cloned into EcoRI and NcoI digested pBACp10H vector that had the IEα^d cloned after the P10 promoter and IEβ^k cloned after the polyhedrin promoter (described in detail below). The nucleic acid molecule having sequences encoding a moth cytochrome C (91-103) peptide, a linker and an IEβ^k gene encoding the IEβ^k β1 and β2 domains is referred to as N-IE^{kd}-MCC.

Using PCR amplification, a hybrid IAβ^d nucleic acid molecule was produced containing sequences encoding amino acid residues 327-339 of chicken ovalbumin (cOVA Peptide (327-339), a linker containing a thrombin cleavage site, and the IAβ^d gene. The hybrid molecule was prepared as

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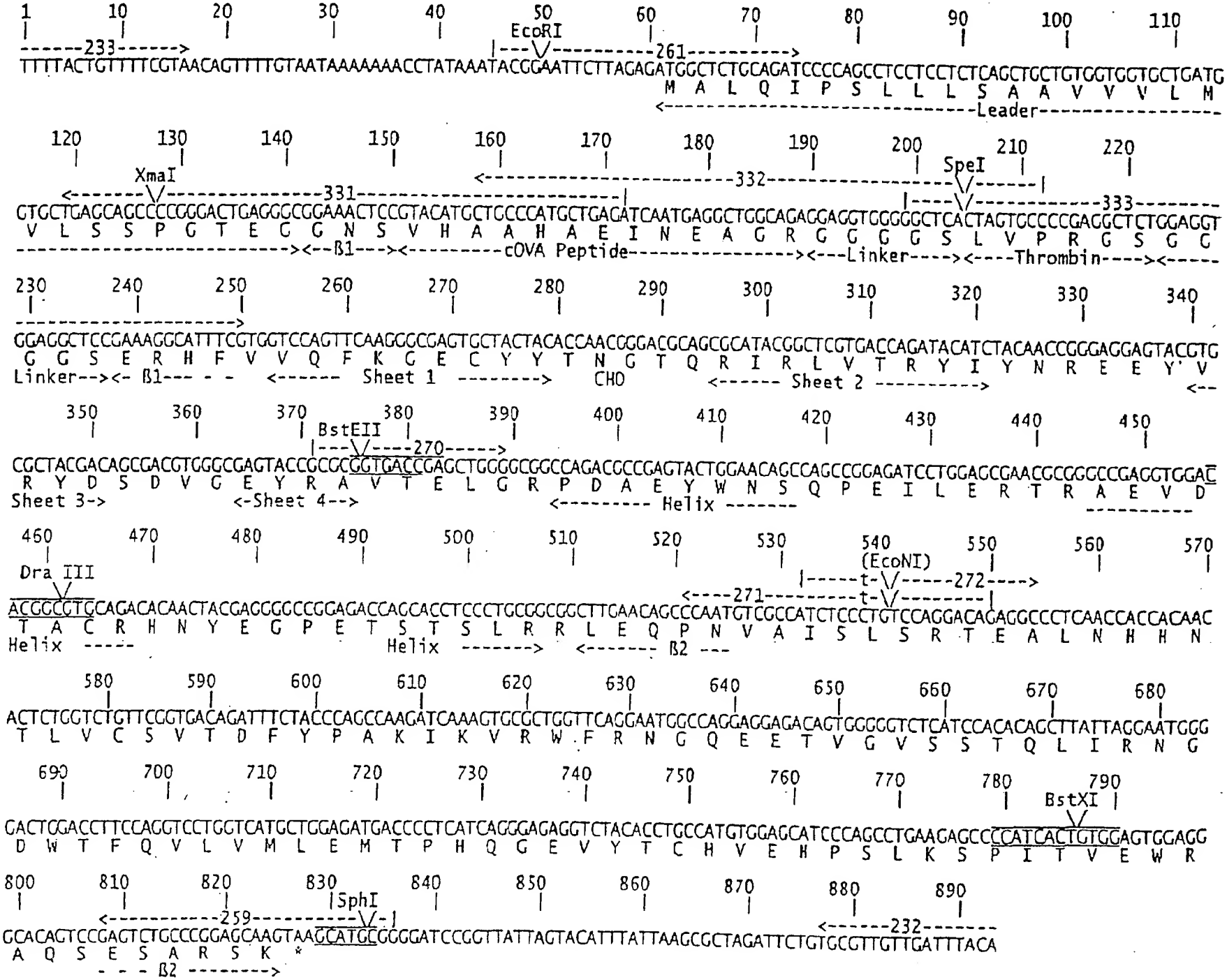
Table 3.

Aßd with Chicken Ovalbumin peptide



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peptide, a linker and an IAβ^d protein including the β1 and β 2 domains is referred to as N-IA^d-OVA

Table 4 lists the nucleic acid and protein sequences of the IEβ^k-MCC and IAβ^d-OVA from the promoters (described below), through the leaders, peptides and linkers, and into the ß1 domains. Similar sequences are shown for IEβ^k or IAβ^d without nucleic acid sequences encoding antigenic peptide or linker.

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The nucleic acid sequences encoding the α and β chains of IE and IA (IEα^d, IEβ^k, IEβ^k-MCC, IAα^d, IAβ^d, and IAβ^d-OVA sequences) were cloned into the P10 and polyhedrin MCS's of the dual promoter baculovirus transfer vector, pBACp10H (Pharmingen). The vector was altered using oligonucleotides and PCR amplification to replace both the EcoRI and BglII sites after the PI0 promoter and the BamHI site after the polyhedrin promoter with new MCS's. The DNA sequences through the new MCS's were:

```
                    XhoI   BstEII     MroI     KpnI
P10- CACTGATCCTCGAGGGGGTGACCGGTCCGGAGGGGTACCAATTCCAG

                    EcoRI    SalI    BglII     SphI    BamHI
Polyhedrin-AAATACGGAATTCGGGTCGACGGAGATCTGGGCATGCGGGGATCCGG
```

To form the recombinant molecule pIE^{kd}-MCC encoding an IE^{kd} $\alpha\beta$ dimer containing the MCC peptide and the linker, the gene encoding the IEα^d protein was ligated into the XhoI and KpnI sites following the P10 promoter, and the IEβ^k-MCC nucleic acid molecule was ligated into the EcoRI and NcoI sites after the polyhedrin promoter. The same procedure was performed to ligate the gene encoding the IEβ^k protein into pBACp10PH to form the recombinant molecule pIE^{kd} encoding an IE^{kd} $\alpha\beta$ dimer.

To form the recombinant molecule pIA^d-OVA encoding an IA^d $\alpha\beta$ dimer containing the cOVA peptide and the linker, the gene encoding the IAα^d protein was ligated into the EcoRI and SphI sites after the polyhedrin promoter and the IAβ^d-OVA nucleic acid molecule was ligated into the EcoRI and

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results showed in all cases equimolar α and ß-chain in the eluted protein indicating initial secretion of αß heterodimers. The overall yield was 0.5 to 1.5mg/liter of culture media.

The stability of the immunoaffinity purified $\alpha\beta$ dimers were analyzed by HPLC gel filtration. For gel filtration analysis, ~50ug of each protein in 20ul was loaded on a Shodex Protein KW-804 HPLC gel filtration column of dimensions 8mm x 300mm (total volume ~15ml). The column was eluted at 0.5ml/min. in PBS and the OD_{280} of the eluate followed. The four elution profiles shown were normalized to the same total OD_{280}. Referring to Fig. 2, the elution positions of molecular weight standards are shown at the top of the Figure: bovine serum albumin, 67kD; ovalbumin, 43kD; and chymotypsinogen, 25kD. In Fig. 2a, IE^{dk} is represented as O--O; $IE^{k/d}$-MCC is represented as ●--●. In Fig. 2b, IA^{d} is represented as O--O; and IA^{d}-OVA is represented as ●--●.

The results shown in Fig. 2b indicate that both $IE^{k/d}$ and IA^{d} protein showed high molecular weight aggregates and apparent size heterogeneity even in the $\alpha\beta$ dimer peak. IA^{d} appeared particularly unstable with considerable dissociation by free α and β chains after elution from the immunoaffinity column. The lack of stability of the $IE^{k/d}$ and IA^{d} proteins is believed to be, in large part due to the lack of antigenic peptide bound to the binding groove of the two proteins.

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immobilized $IE^{k/d}$-MCC and IA^d-OVA protein. Immunoaffinity purified IE^{dk}, IA^d, IEdk-MCC, and IA^d-OVA were prepared as described above. In addition, an attempt was made to produce IE^{dk} and IA^d MHC-peptide complexes by mixing: (1) synthetically produced MCC (88-103) peptide mixed with IE^{dk} protein (IE^{dk} + MCC); and (2) synthetically produced OVA (327-339) was mixed with IA^d protein (IA^d + OVA). The mixtures were incubated overnight in 50μl of citrate buffer at pH 5.0, at 37°C and at a concentration of 10μg of the Class II molecules and 10μg of the peptide. Following incubation, the mixtures were neutralized and unbound peptide was removed using a Centricon 10 filter unit.

Aliquots of the $IE^{k/d}$-MCC and IA^d-OVA protein were digested by a 2 hour incubation of 10μg of the complex with 2×10^{-3} units of thrombin at pH 6.6. Such conditions digested at least 80% of the complex as assessed by SDS-PAGE. Different amounts of the various Class II preparations were immobilized by overnight non-specific adsorption to the bottom of wells of 96 well Immulon II plates. Either 5KC-73.8 (IE^{dk} + MCC specific) (5KC) or DO-11.10 (IA^d + OVA specific) T cell hybridomas (10^5 cells) were added in 250ul of tissue culture medium to each coated well of the 96-well plate. The plates were cultured overnight and the amount of IL-2 produced by the hybridomas was assessed. The results are shown in Fig. 3. Fig. 3a shows the results of IL-2 production by 5KC-73.8 cells in the presence of IE^{dk}-MCC (●), IE^{dk} + MCC (■), thrombin

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covalently associated Peptide-MHC complex induces a better immune response than complexes formed by mixing the peptide with the MHC protein in solution. The results also indicate that the covalently associated Peptide-MHC complex is more stable than an MHC protein in the absence of peptide. In both cases treatment of the peptide/MHC covalent complex with thrombin to cleave the linker between them only modestly improved T cell hybridoma recognition. This indicates that the linker most likely extends from the C-terminal end of the peptides around the side rather than over the top of the Class II α chain α helix in order to reach the N terminal end of the Class II β chain (see Fig. 1).

Together all these data show that genes coding for peptides covalently associated to MHC Class II proteins by a linker can be expressed as soluble proteins capable of being recognized by T cells. The data also indicate that the peptides linked to the MHC protein form stable complexes more effective at stimulating a T cell response than complexes formed by mixing the peptide with the MHC protein.

Example 2

This Example demonstrates that genes coding for peptides covalently associated to MHC Class II proteins by a linker can be expressed as stable membrane-bound proteins capable of being recognized by T cells.

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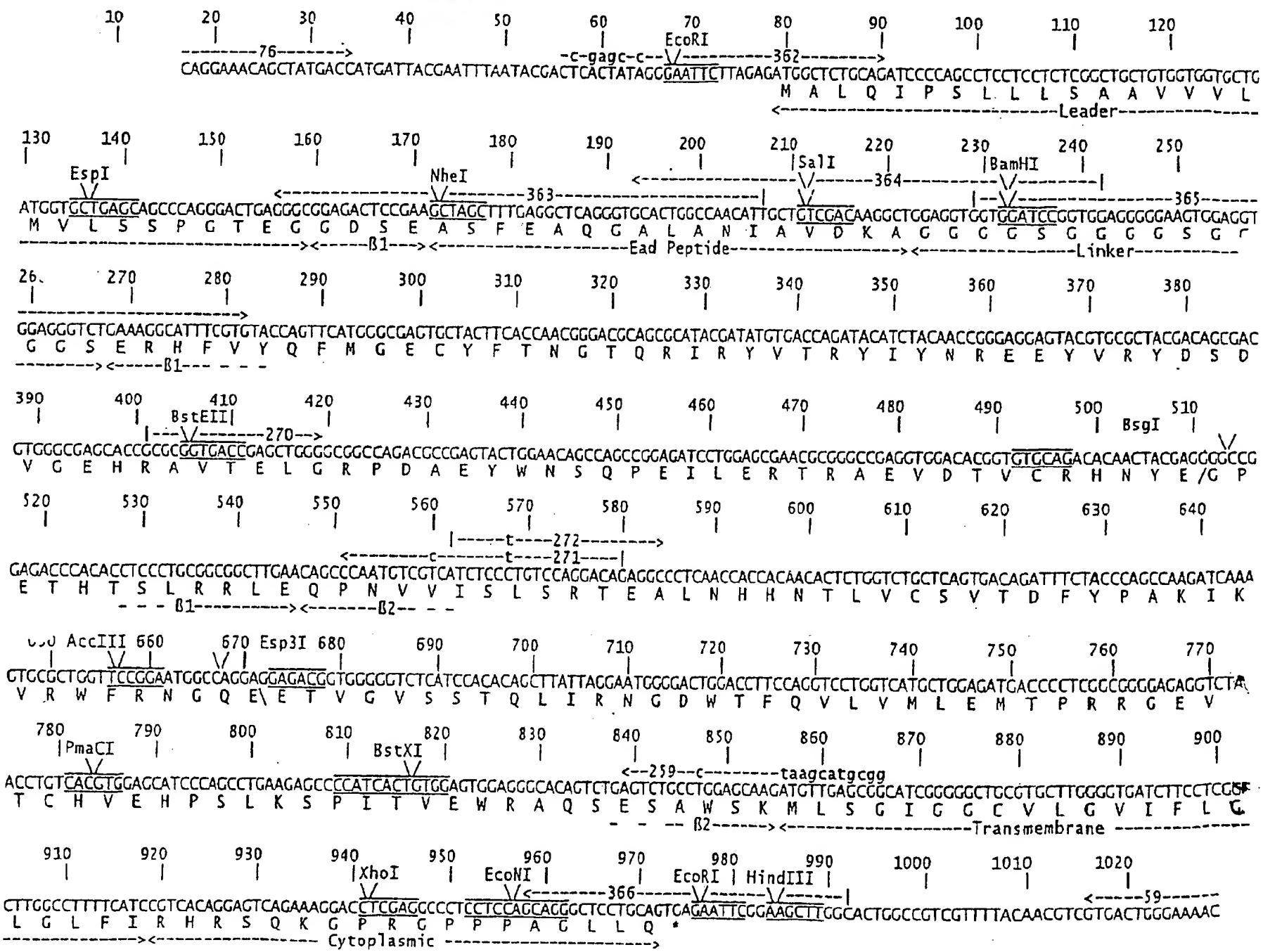
Table 5.

Aßb with Ead Peptide in pTZ18R



been digested with BamHI and HindIII to form a pTZ-IA^b-Ea construct. The pTZ-IA^b-Ea construct was then digested with EcoRI and subcloned into the expression vector pDOI-5 (obtained from D. Mathis and C. Benoit, containing a class II promoter and enhancer region, a β-globin intron and EcoRI cloning site) to form the recombinant molecule pM12-IA^b-Ea encoding IA^b-Ea protein. pM12-IA^b-Ea recombinant molecules having sequences encoding IA^b-Ea protein in the correct orientation were identified by nucleic acid sequencing multiple pM12-IA^b-Ea recombinant molecules.

The recombinant molecule pFIB-IA^b-Ea was produced for transfection into fibroblast cells by subcloning the pTZ-IA^b-Ea construct described above with EcoRI and subcloning the EcoRI fragment into EcoRI digested expression vector pHβAcPr-1-neo (obtained from S. Hedrick, containing the human β-actin promoter and enhancer, and a sequence encoding neomycin resistance). The orientation of each cloned insert was determined after cloning by nucleic acid sequencing.

Table 6 lists the nucleic acid and protein sequence of the IA ß chain from the leader, through the peptide and linker, into the ß1 domain. A similar construction was made for IAβ^b without nucleic acid sequences encoding antigenic peptide as controls.

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A B cell line M12.C3 was transfected with the recombinant molecule pM12-IA^b-Ea or a recombinant molecule encoding an IA^b protein. Using standard fluorescence activated cell sorter analysis (FACS analysis), two different antibodies were used to detect expression of IA^b-Ea protein on the surface of the M12 cells, an anti-IA^b antibody and an antibody (5A) specific for IA^b protein bound by IEα^d peptide. The results shown in Fig. 4 indicate that the M12.C3 cells transfected with IA^b-Ea protein react well with both antibodies. Untransfected M12.C3 cells and normal spleen cells are shown as negative controls. Thus, the IA^b-Ea protein is expressed in M12.C3 cells and reaches the cell surface. Moreover, the covalently bound peptide is bound to the peptide-binding groove of the IA^b protein as indicated by the binding of the 5A antibody.

An experiment was performed to determine the extent of binding of the covalently associated IEα^d to the binding site of the associated IA^b protein. M12.C3 cells bearing either IA^b-Ea protein or IA^b protein were mixed with T hybridoma cells specific for IA^b protein bound by cOVA peptide (BO.97.10). Increasing concentrations of either cOVA peptide (described in Example 1) or ovalbumin was added to the mixture and the amount of IL-2 produced by the hybridoma was measured. The cOVA peptide is known to be capable of binding to the binding site of IA^b protein. Thus, the cOVA peptide will bind to any IA^b protein not bound by peptide. The IL-2 production is a measurement of

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The ability of IA^b-Ea protein bearing M12.C3 cells to present peptide was tested by measuring IL-2 production by a T cell hybridoma specific for IA^b-Ea protein. M12.C3 cells bearing IA^b-Ea protein or IA^b protein (10^5 cells/well) were mixed with BE-20.6 T hybridoma cells (10^5 cells/well) and cultured for 24 hours. Following incubation, the amount of IL-2 produced by the hybridomas was measured. The results indicate that IA^b-Ea protein M12.C3 cells were capable of inducing IL-2 production by the hybridoma while IA^b protein bearing M12.C3 cells were not capable of inducing IL-2 production (see Fig. 6). Thus, the covalently bound peptide is bound to the peptide-binding groove of the IA^b protein and expressed on the surface of the B cell in a manner such that the complex can induce a T cell response.

In a second experiment, fibroblasts were transfected with the recombinant molecule pFIB-IA^b-Ea or a recombinant molecule encoding an IA^b protein were transfected into fibroblast cells. Expression of IA^b-Ea protein on the surface of the fibroblasts was analyzed by FACS analysis using the same antibodies described above. As shown in Fig. 7, the fibroblasts transfected with the recombinant molecule encoding IA^b-Ea protein react well with both anti-IA^b antibody and antibody specific for IA^b protein bound by $IE\alpha^d$ peptide (5A). Thus, the proteins encoded by the transfected IA^b-Ea recombinant molecule were well expressed

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production. These results demonstrate that MHC protein that has a peptide covalently associated via a linker, can be expressed in a cell type that does not typically express MHC protein, and that the Peptide-MHC complex can be recognized by a hybridoma, resulting in stimulation of IL-2 production.

Together all these data show that genes coding for peptides linked to MHC Class II proteins can be expressed as membrane-bound protein in cells. The covalently bound peptide can bind to the groove of the MHC protein and be recognized by antibodies or T cells.

Example 3

This example demonstrates that a gene encoding peptides covalently associated to an MHC Class II protein by a linker can be expressed as a transgene in mice.

A. Production of IEß^k-MCC Transgenic Mice

A nucleic acid molecule was produced using residues 91-103 of moth cytochrome c (MCC Peptide (91-103)), linker, and IEß^k encoding genes described in detail in Example 1 and was subcloned behind a hemoglobin β-chain promoter. The resulting recombinant molecule is referred to herein as pIEß^k-MCC-Tg. Numerous copies of pIEß^k-MCC-Tg were injected into the nuclei of fertilized eggs removed from (Bl/6xSJL)F2 mice and the eggs replaced into mothers. Two mice were identified that expressed the IEß^k-MCC transgene. These mice are referred to as founders. Transgenic progeny

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found on the surface of later red blood cell precursors and red blood cells but not on other hematopoietic cells.

Using standard fluorescence activated cell sorter techniques (FACS), populations of bone marrow or spleen cells were incubated with fluorescein-labelled anti-Ter 119 antibodies using standard conditions known in the art. The labelled cells were then sorted by FACS to obtain cells having bound anti-Ter 119 antibody. The FACS sorted Ter 119 positive transgene positive cells were then assayed for their ability to stimulate T cell hybridomas specific for IE^k with bound MCC 91-103 peptide using the method described in Example 1.

The results indicate that the Ter 119 positive cells stimulated T cells specific for IE^k with bound MCC 91-103 peptide in the absence of added MCC 88-103 peptide (see Fig. 11). Thus, the transgenic IE^k-MCC protein is expressed on later red blood cell precursors and red blood cells contained in the populations of bone marrow and spleen cells isolated from the transgenic H-2^k mice.

Example 4

This example demonstrates that IE^k protein covalently attached to MCC 91-103 reduces the number of T cells specific for IE^k with bound MCC 91-103 peptide in mice.

Transgenic progeny expressing transgenic IE^k-MCC protein were crossed with a strain of mice expressing H-2^k and a transgenic T cell receptor that recognizes (i.e.,

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Transgenic progeny expressing transgenic IE^k-MCC protein were crossed with a strain of mice expressing IE^k and IE^s. Non-transgenic IE^s mice, when primed with MCC 91-103 peptide, produce T cells that can respond to the MCC 91-103 peptide bound to IE^k, as well as, the same peptide bound to IE^s. Such T cells, however, comprise two different sets of T cells. IE^k/IE^s (kxs) mice and IE^k/IE^s (kxs) mice transgenic for IE^k-MCC were primed with MCC 91-103 peptide, or a positive control hemoglobin 64-76 peptide (Hbβd) that can also be recognized by T cells in the presence of either IE^k or IE^s. T cells were isolated from the primed mice and assayed for their ability to respond to MCC 88-104 peptide or Hbβd 64-76 peptide plus IE^k or IE^s using methods described in Example 1.

The results are shown in Fig. 13 and indicate that T cells isolated from mice expressing the transgenic IE^k-MCC protein responded well to the Hbβd 64-76 peptide plus IE^k or IE^s. The T cells also responded well to the MCC 88-104 plus IE^s. When, however, the response of T cells isolated from mice expressing the transgenic IE^k-MCC protein to MCC 88-104 IEk was compared with the response of T cells isolated from normal kxs mice, the transgenic T cells responded poorly. Thus, the results indicate that the transgenic IE^k-MCC protein induced tolerance in the IE^k-MCC transgenic mice.

While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will

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What is claimed is:

1. A Peptide-L-MHC molecule comprising an antigenic peptide, a linker and an MHC segment, wherein said peptide is linked to said MHC segment by said linker.

2. The Peptide-L-MHC molecule of Claim 1, wherein said MHC segment comprises at least a portion of an MHC class II protein.

3. The Peptide-L-MHC molecule of Claim 1, wherein said peptide is capable of binding to a peptide binding site of an MHC protein.

4. A formulation capable of inducing T cell tolerance comprising a composition selected from the group consisting of a Peptide-L-$MHC_{\alpha+\beta 2m}$ composition and a Peptide-L-$MHC_{\alpha+\beta}$ composition having an antigenic peptide joined by a linker to an MHC segment, said composition being anchored to the plasma membrane of a cell essentially incapable of stimulating a T cell response.

5. The formulation of Claim 4, wherein said cell is selected from the group consisting of red blood cells, fibroblasts, pluripotent progenitor cells, epithelial cells and neural cells.

6. A nucleic acid molecule having a sequence encoding a Peptide-L-MHC molecule comprising an antigenic peptide joined by a linker to an MHC segment.

7. The nucleic acid molecule of Claim 6, wherein said MHC segment is encoded by at least a portion of an MHC class II β chain gene.

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the group consisting of red blood cells, antigen presenting cells, fibroblasts, pluripotent progenitor cells, epithelial cells and neural cells.

14. The method of Claim 13, wherein said cells comprise red blood cells.

15. The method of Claim 11, wherein said animal is tolerized by said pharmaceutical reagent.

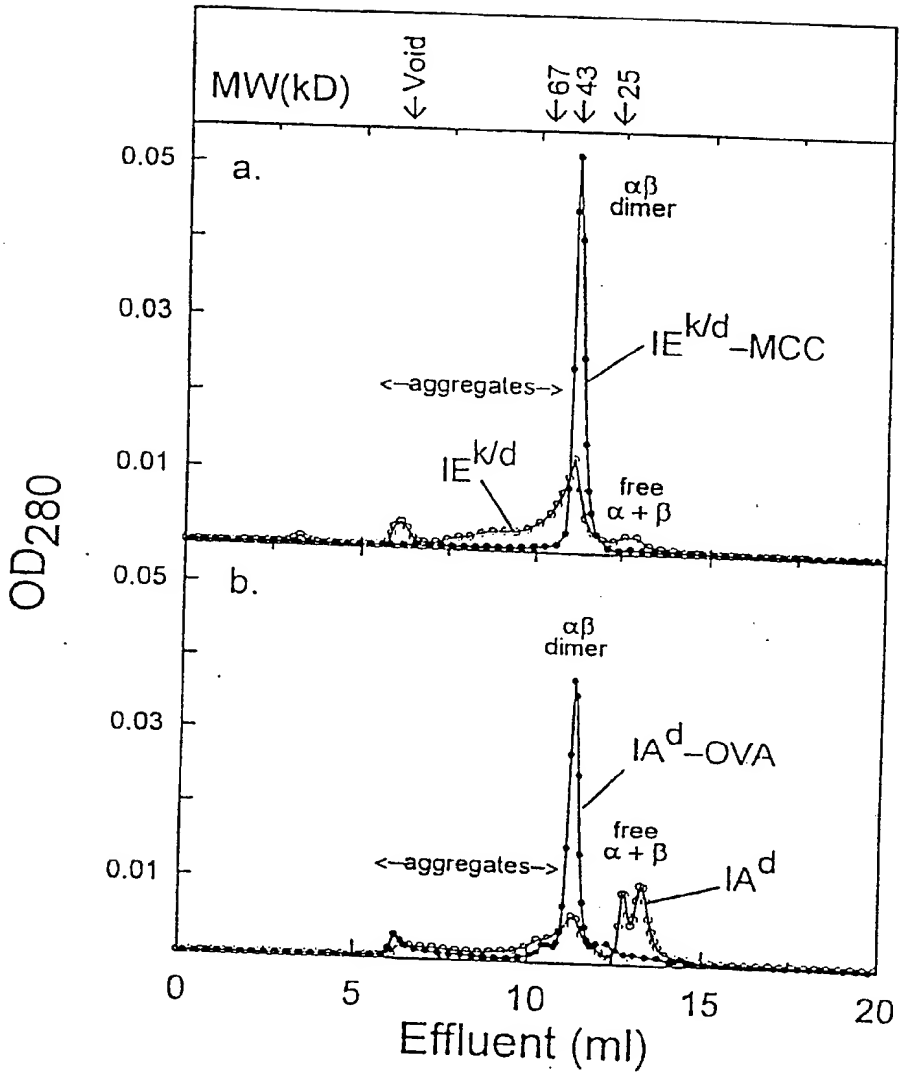

Fig. 2

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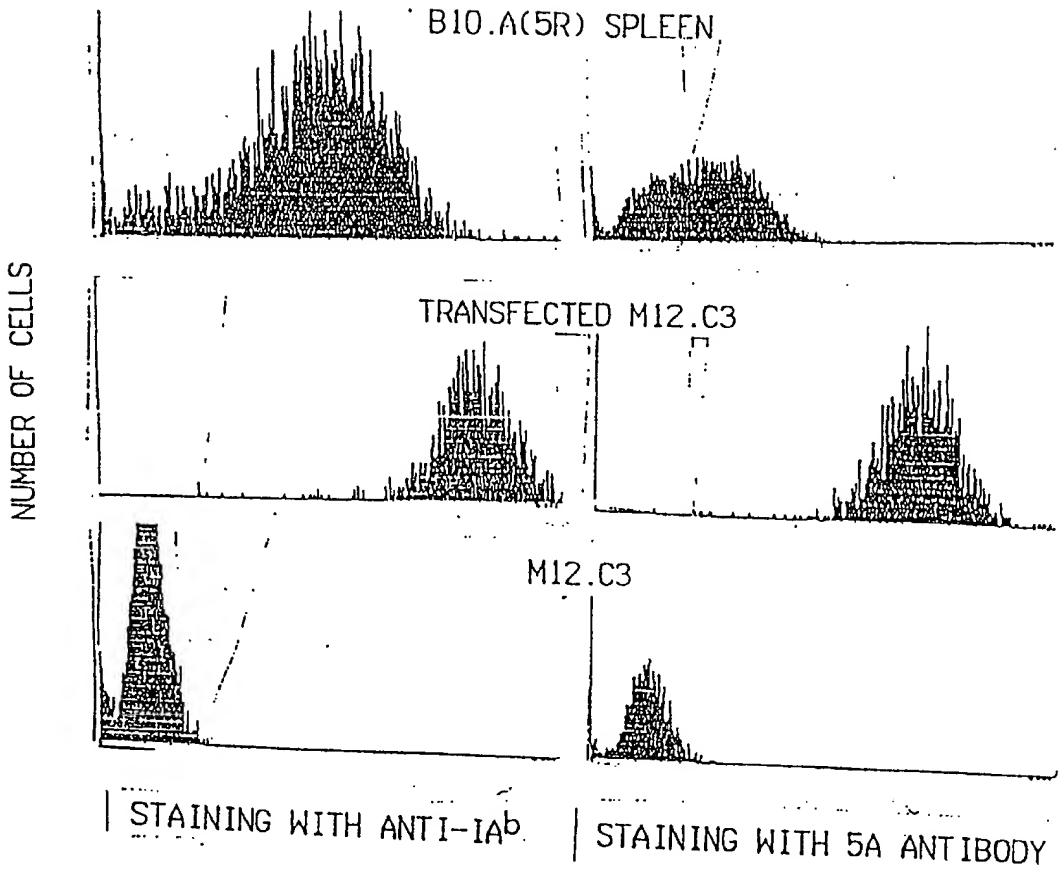

Fig. 4

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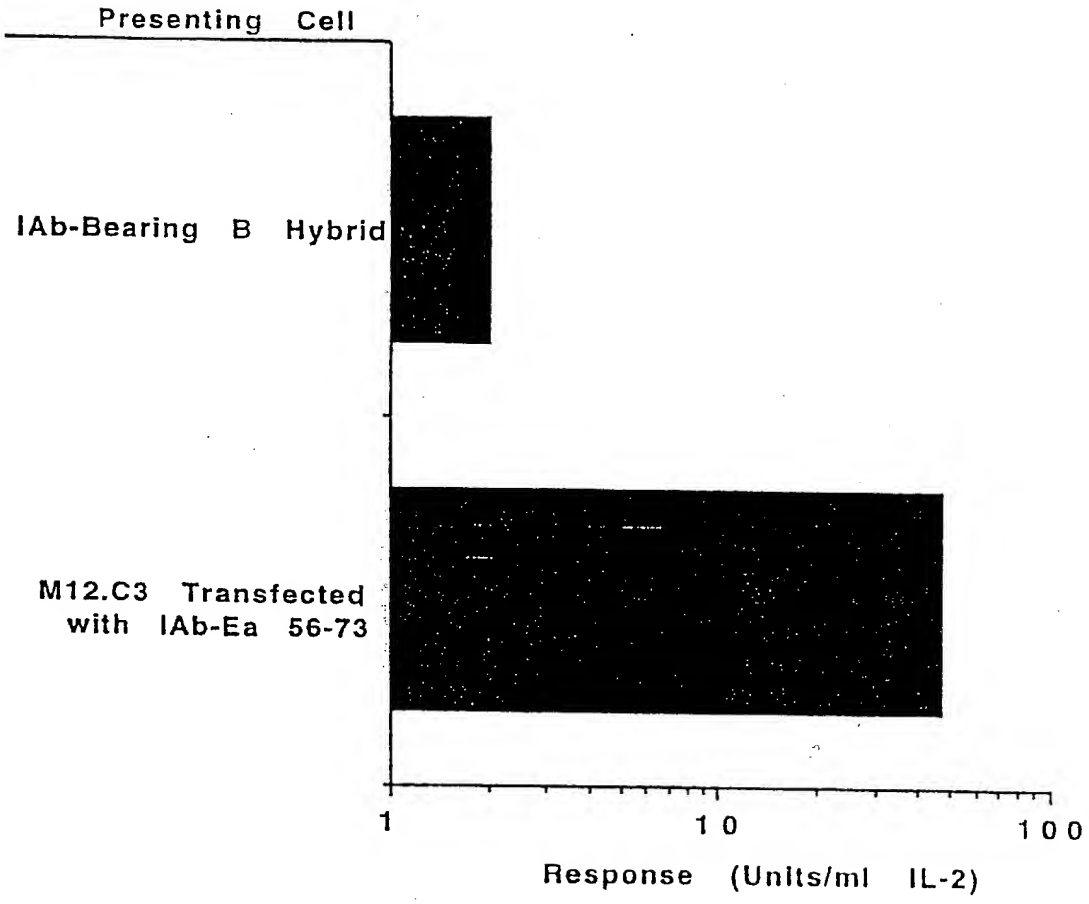

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Fig. 6

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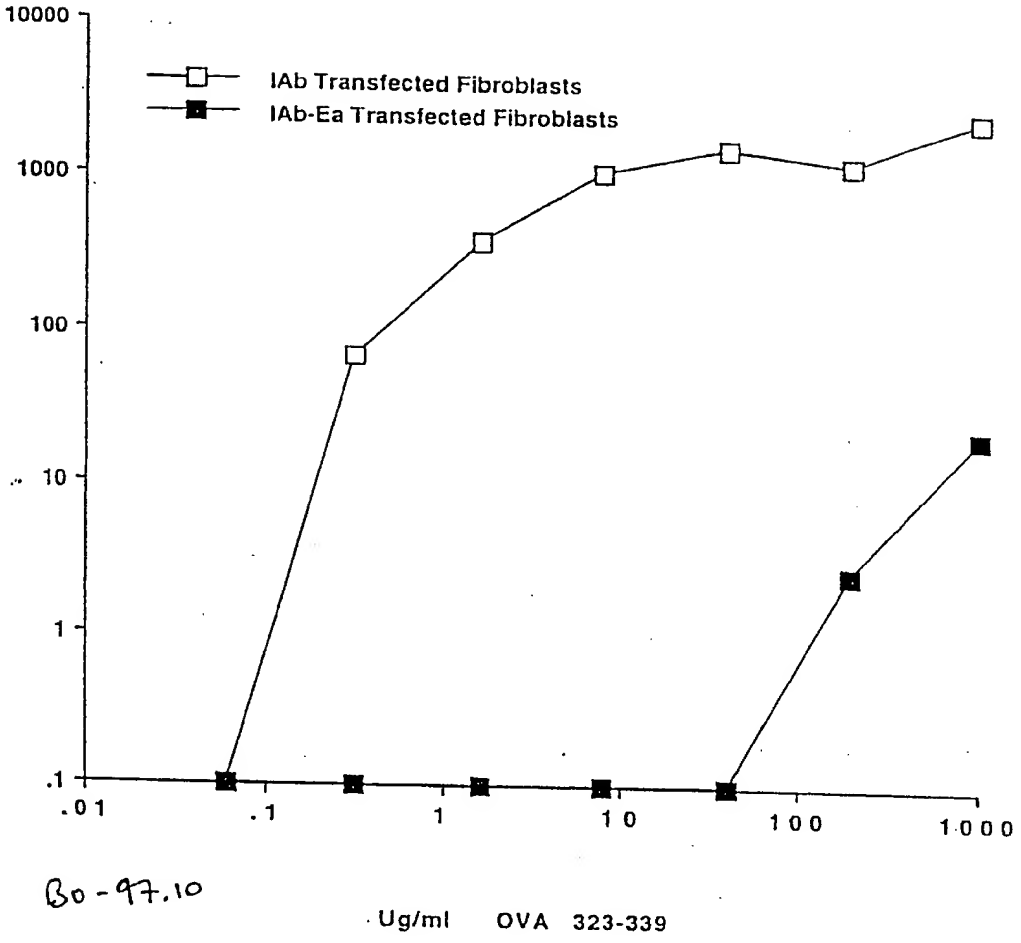

Fig. 8

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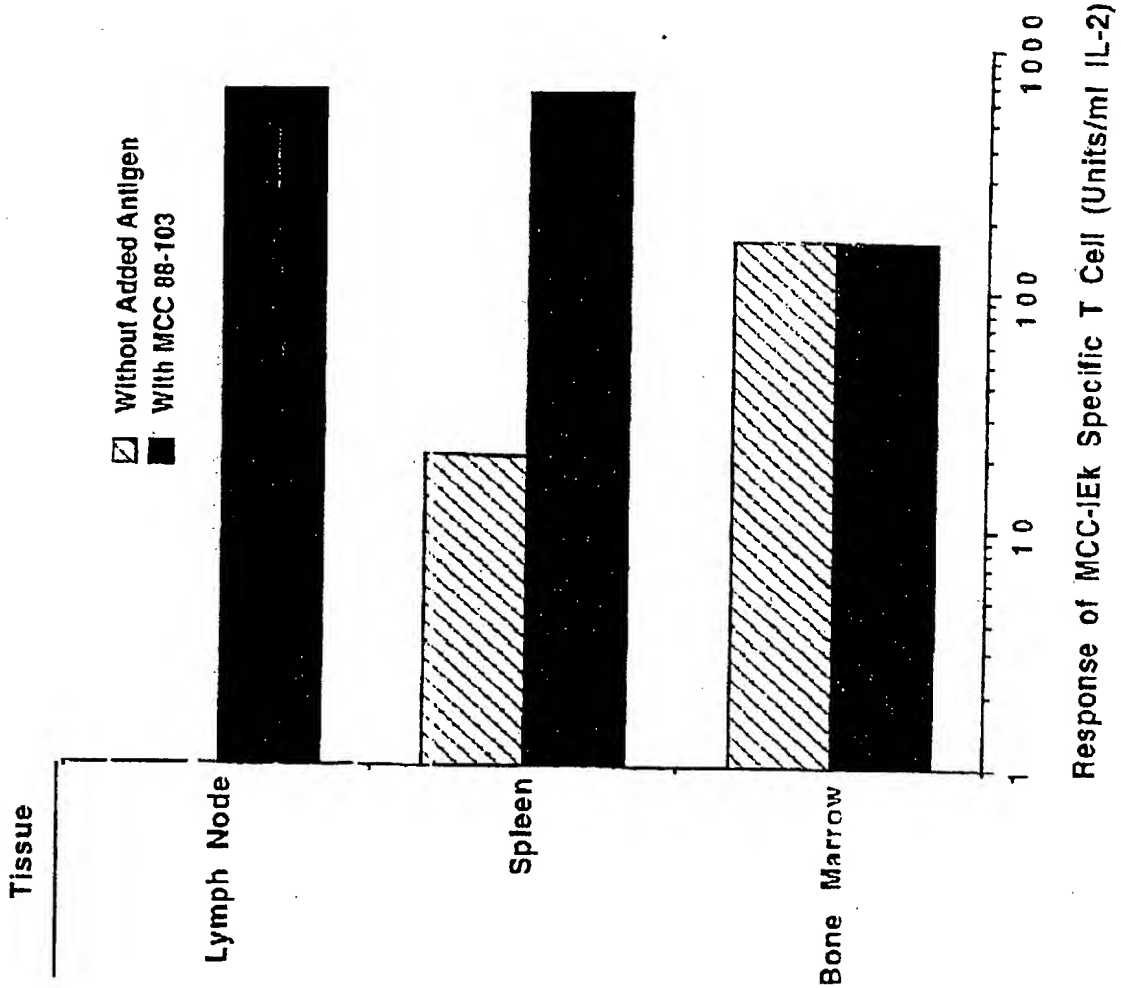

FIG. 10

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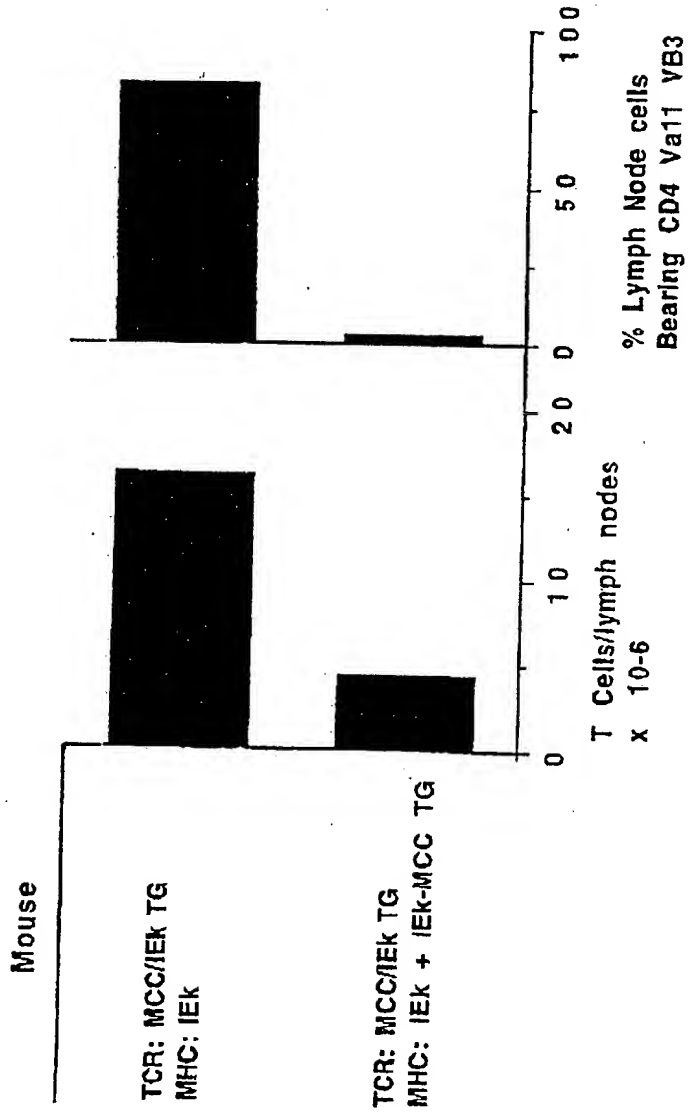

FIG. 12

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/02689

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 19/00; A61K 35/12, 35/18, 39/00, 39/385; C12N 15/62, 5/10
US CL :530/350, 403; 424/192.1, 93.21; 536/23.4; 435/240.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 403; 424/192.1, 93.21; 536/23.4; 435/240.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

U.S. Automated Patent Search, World Patents Index, Medline. Keywords: MHC, fusion, chimer?, chimaer?, conjugat?, biomembrane#, red blood cell#, rbc.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 5,242,687 (TYKOCINSKI ET AL) 07 September 1993, see column 2, lines 39-50; column 6, lines 21-43; column 11, line 59 to column 12, line 59; column 13, line 61 to column 14, line 7; and column 17, lines 27-39.	1-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
05 JUNE 1995

Date of mailing of the international search report
13 JUN1995

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